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<b>TRANSMITTAL LETTER TO THE UNITED STATES          DESIGNATED/ELECTED OFFICE (DO/EO/US)          CONCERNING A FILING UNDER 35 U.S.C. 371</b>				U.S. APPLICATION NO. (If known, see 37CFR1.5) <b>10/019409</b>	
INTERNATIONAL APPLICATION NO. PCT/JP00/02112		INTERNATIONAL FILING DATE March 31, 2000		PRIORITY DATE CLAIMED June 29, 1999	
TITLE OF INVENTION <b>SULFUR ATOM-FREE ENZYME PROTEIN</b>					
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Applicant(s) herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information					
1.	<input checked="" type="checkbox"/>	This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.			
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		a.	<input type="checkbox"/>	is attached hereto (required only if not communicated by the International Bureau.	
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7.	<input checked="" type="checkbox"/>	Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)).			
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12.	<input checked="" type="checkbox"/>	An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.			
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DATED: December 28, 2001

## SULFUR ATOM-FREE ENZYME PROTEIN

### FIELD OF THE INVENTION

The present invention relates to a sulfur atom-free enzyme protein.

### BACKGROUND ART

Enzymes, because of their very high substrate specificity, has been attempted and expected to be used, for example, in development of analytical instruments such as biosensors, in fine chemical industries such as bioreactors, and in decomposition and removal of specific pollutants.

Recent development of mass spectrometers has enabled mass spectrometry of proteins. When determining the mass number of proteins by mass spectrometry, even in proteins that are highly purified as proteins, existence of proteins having a mass number other than that expected from the amino acid sequence has been found. For example, as a result of detailed inspection using L-cystein-free dihydrofolate reductase, it was found that the change in mass number was based on a unit of the mass number 16, and the main cause thereof is oxidation of methionine.

When considering the utilization of enzymes, denaturation of proteins caused by oxidation becomes a serious drawback. For example, many biosensors make use of an electrode reaction, and in this case, oxides such as hydrogen peroxide are produced due to the electrode reaction. The oxides could oxidize enzymes, thereby accelerating deterioration of the entire sensor or reducing reliability. When an enzyme is used in a solution for a long period of time, the prevention of oxidation by oxygen in an aqueous solution is difficult or problematic from a viewpoint of cost.

There are 5 types of atom which constitute proteins: hydrogen (H); carbon (C); nitrogen (N); oxygen (O); and sulfur (S). Among them, the sulfur atom has greater reactivity such as valence properties, in comparison with other atoms.

Proteins produced by organisms are constituted by 20 types of L-amino acid residues: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; L-tryptophan; L-cystein; and L-methionine; which are coded by triplet codons in DNA of genes. Among them, L-cystein and L-methionine are sulfur-containing amino acids.

The sulfur atom of L-cystein exists as a thiol (-SH) group. The thiol group has a very high reactivity, is easily oxidized by oxygen and hydrogen peroxide, and produces disulfide, and further sulfinic acid.

The sulfur atom of L-methionine exists as a thioether (-SCH<sub>3</sub>) group. The thioether group has reactivity not as strong as that of the thiol group, however, it is easily oxidized by hydrogen peroxide to produce methionine sulfoxide.

This suggests that an enzyme which does not include L-cystein and L-methionine, i.e., one which is free from sulfur-containing amino acids, possesses antioxidative properties.

There are 3 types of codon corresponding to L-cystein and L-methionine, that is, TGT and TGC (L-cystein) and ATG (L-methionine). In contrast, there are 58 types of codon corresponding to other 18 types of sulfur-free L-amino acid. Therefore, proteins contain L-cystein or L-methionine at a ratio of 1 in every 20 amino acids (3/61) on average. This indicates that proteins constituted by 100 or more amino acids contain L-cystein or L-methionine with a very high probability. In fact, among the proteins which have hitherto been reported, when enzymes having catalytic functions are inspected, enzymes free from L-cystein and L-methionine are not found.

That is, all enzymes produced by organisms contain sulfur-containing amino acids.

On the other hand, results of protein mutation based on recent genetic manipulation demonstrate that at least in the case of mutation of one site, it is possible to substitute sulfur-containing amino acids by other amino acids without loss of original enzyme function. Before the present invention was completed, it had not been clarified whether or not enzymes, in which all sulfur-containing amino acids were substituted with other amino acids, had activities equivalent to the original functions. Rather, it was the dominant point of view that sulfur-containing amino acids were essential to expression of enzyme activity.

This suggests the following two opposing possibilities.

The first possibility is that the presence of the sulfur-containing amino acids is essential to the development of very high enzyme activity necessary for organisms to maintain their lives.

The second possibility is that the presence of the sulfur-containing amino acids is not necessarily essential to the development of very high enzyme activity. However, in the origins of life, proteins had happened to be utilized which contained sulfur atoms therein and the form for coding genes was established, and thus, enzymes produced by organisms contain sulfur-containing amino acids corresponding to the frequency with which the codon was used. Therefore, enzymes can be prepared in which all the sulfur-containing amino acids are substituted with other amino acids, and which have equivalent functions to organism-produced enzymes containing sulfur-containing amino

acids.

If the second possibility were correct, a general process for preparing enzymes having antioxidation properties can be developed wherein all the sulfur-containing amino acids of enzymes which contain organism-derived sulfur-containing amino acids are substituted with other amino acids.

#### DISCLOSURE OF THE INVENTION

The present inventors have keenly studied and substantiated that, an enzyme which has enzyme activity equivalent to or greater than the original enzyme, can be prepared even where all sulfur-containing amino acids of an enzyme which contains organism-derived sulfur-containing amino acids, are substituted with other amino acids. The present inventors further established a strategy to vary an organism-derived wild type enzyme to an enzyme free from sulfur-containing amino acids. This has led to the completion of the present invention.

According to the present invention, a wild type enzyme includes enzymes obtained by artificially varying organism-derived enzymes in addition to the organism-derived enzymes.

More specifically, the object of the present invention is to provide an enzyme protein which retains the activity of a wild type enzyme while having an antioxidation property against oxidation by hydrogen peroxide and the like and a process for producing the same.

In order to attain the above object, the present invention adopts the following constitution.

1. A sulfur atom-free enzyme protein constituted by 18 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; and L-tryptophan.

2. The sulfur atom-free enzyme protein according to 1 above which retains the activity of the original enzyme protein and has oxidation resistance, wherein L-cystein and L-methionine residues in enzyme proteins constituted by 20 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; L-tryptophan; L-cystein; and L-methionine are, substituted with 18 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine;

L-threonine; L-valine; L-tyrosine; and L-tryptophan.

3. The sulfur atom-free enzyme protein according to 2 above wherein amino acid substitution is carried out by site-directed mutagenesis using synthetic DNA.

4. The sulfur atom-free enzyme protein according to any one of 1 to 3 above wherein the enzyme activity is oxidation-reduction activity or hydrolysis activity.

5. The sulfur atom-free enzyme protein according to any one of 1 to 4 above, which retains the activity of dihydrofolate reductase and has oxidation resistance.

6. The sulfur atom-free enzyme protein according to any one of 1 to 4 above, which retains the activity of xylanase and has oxidation resistance.

7. A method of producing a sulfur atom-free enzyme protein prepared by combined mutation comprising the following steps:

- (1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein consisting of a total length of m amino acids, and comprising n number of sulfur atom-containing amino acids (sulfur-containing amino acids), wherein a position of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), by L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline codon, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting the protein with the highest activity, thereby obtaining a substitution mutant A1/MA1;
- (2) preparing a mutant gene in which codons encoding sulfur-containing amino acids at another sites  $A_i$  ( $i = 2$  to  $n$ ) are substituted with codons encoding another amino acids among the 18 types of amino acid according to 1 above (hereinafter referred to as "another amino acid other than the sulfur-containing amino acid"), expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting p number of mutant enzyme proteins having enzyme activity, thereby obtaining substitution mutants  $A_i/B_{ij}$  ( $j = 1$  to  $p$ );
- (3) selecting a maximum of 3 substitution mutants having the highest activity  $A_i/B_{i1}$ ,  $A_i/B_{i2}$ , and  $A_i/B_{i3}$ , wherein activity decreases in order,  $A_i/B_{i1} > A_i/B_{i2} > A_i/B_{i3} \dots > A_i/B_{ip}$ ; and
- (4) selecting substitution mutants having activity in respect of sulfur-containing amino acids at all sites  $A_i$  ( $i = 2$  to  $n$ ) in the same manner as (2) and (3) above, preparing a maximum of  $3 \times (n-1)$  mutants being all combinations of these mutants with the mutant of A1/MA1, measuring the enzyme

activity thereof, and obtaining a mutant enzyme protein having activity greater than or equivalent to the original enzyme protein.

8. A method of producing a sulfur atom-free enzyme protein prepared by stepwise mutation comprising the following steps:

- (1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein consisting of a total length of  $m$  amino acids, and comprising  $n$  number of sulfur atom-containing amino acids (sulfur-containing amino acids), wherein a position of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), by L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline codon, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting the protein with the highest activity, thereby obtaining a substitution mutant A1/MA1;
- (2) preparing a mutant gene in which a codon encoding a sulfur-containing amino acid at A2 of A1/MA1 mutant is substituted with a codon encoding the "another amino acid other than the sulfur-containing amino acid", expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained double mutant enzyme protein, and selecting a maximum of 3 triple mutants with the highest activity;
- (3) preparing a mutant gene in which a codon encoding a sulfur-containing amino acid at each A3 of the obtained double mutants is substituted with a codon encoding the "another amino acid other than the sulfur-containing amino acid", expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained triple mutant enzyme protein, and selecting a maximum of 3 triple mutants with the highest activity; and
- (4) in the same manner as described above, preparing a quadruple mutant, ..., multiple number of  $n$  mutant, inspecting enzyme activity of the last multiple number of  $n$  mutant, and preparing a mutant enzyme protein having activity greater than or equivalent to that of the original enzyme.

9. The process for producing a sulfur atom-free enzyme protein prepared by the stepwise mutation according to 8 above wherein the order of stepwise mutation sites is according to any one of ( $n!$  types) permutations and combinations of A1, A2, ..., An.

10. A process for producing a sulfur atom-free enzyme protein wherein, in an enzyme protein consisting of a total length of  $m$  amino acids and comprising  $n$  number of sulfur-containing amino acids, in which a site of a sulfur-containing amino acid on

the sequence is  $A_i$  ( $i = 1$  to  $n$ ), a process according to 7 above is adopted at  $k$  number of sites and a process according to 9 above is adopted at remaining  $n - k$  number of sites.

In the present invention, to retain activity of the original proteins means that the protein does not have not less than 10% activity, preferably not less than 50% activity, particularly preferably not less than 100% activity, of the wild type enzyme, and can be usable for the same applications as the original enzyme proteins.

The procedure of the first process to prepare the intended enzyme protein in the present invention will be described.

It is provided that a wild type enzyme, consisting of a total length of  $m$  amino acids contains  $n$  number of sulfur-containing amino acids. The respective positions of the sulfur-containing amino acids on the amino acid sequence are designated as  $A_i$  ( $i = 1$  to  $n$ ).

The amino acid resulting from the initiation codon of a protein is L-methionine.

To avoid amino terminal L-methionine, methionine can be eliminated from the amino terminus pursuant to the reaction specificity of methionine-aminopeptidase found in the host cell.

For example, when *Escherichia coli* is employed as a host, any of L-methionine-L-alanine, L-methionine-L-serine, and L-methionine-L-proline may be adopted as the amino terminus so that a protein is expressed with eliminating L-methionine from the terminal. Accordingly, at the outset, as a mutation of the amino terminus of the enzyme, mutant genes having codons for L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline are prepared, the prepared mutant genes are expressed in a host, the activity of the obtained mutant is measured, and the mutant with the highest activity level is selected to prepare a mutant enzyme in which the amino terminus is not methionine.

The mutation thus obtained is represented by A1/MA1.

Regarding sulfur-containing amino acids at other sites  $A_i$  ( $i = 2$  to  $n$ ), the codon encoding the sulfur-containing amino acid is substituted with a codon encoding an "amino acid other than a sulfur-containing amino acid" (a maximum of 18 types) to prepare a mutant gene. The obtained mutant gene is expressed in a host cell, and the obtained double mutant enzyme protein is inspected for enzyme activity.

Amino acid substitution at a specific site in an enzyme protein can be carried out by site-directed mutagenesis using synthetic DNA.

As processes for site-directed mutagenesis, many processes are known including a process by Zoller, Smith et al. (Zoller, M.J. and Smith, M. (1983) *Methods in Enzymology*, vol. 100, p. 468) and improvements thereof, and processes utilizing



PCR employed in the present examples and the like. In the present invention, any mutation process may be adopted so far as it can be applied to amino acid substitution of a sulfur-containing amino acid at a target amino acid site, and that object can be accomplished. Therefore, the present invention is not limited by the process for preparing mutants.

When a substitution mutant of sulfur-containing amino acid  $A_i$  is prepared to inspect its activity,  $p$  number of mutants exhibiting activity equivalent to or greater than the wild type enzyme are found. The amino acid is determined as  $B_{ij}$  ( $j = 1$  to  $p$ ) where activity of  $A_i/B_{ij}$  substitution mutant is ordered from highest to lowest. More specifically, activities of the mutants decrease in the order of  $A_i/B_{i1} > A_i/B_{i2} > A_i/B_{i3} > \dots > A_i/B_{ip}$ .

Among  $A_i/B_{ij}$  substitution mutants, a maximum of 3 substitution mutants having the highest activity are selected, specifically,  $A_i/B_{i1}$ ,  $A_i/B_{i2}$ , and  $A_i/B_{i3}$ .

Selection is carried out in the same manner for all  $i$  ( $i = 2$  to  $n$ ) to prepare a maximum of  $3 \times (n - 1)$  mutants being all combinations of these mutations with mutation  $A_1/MA_1$ . The activities of  $3 \times (n - 1)$  mutants are then inspected to select the mutant having activity equivalent to or greater than that of the wild type enzyme.

It is obvious that the mutant enzymes thus obtained are free of sulfur-containing amino acids.

The enzymes free from sulfur-containing amino acid thus obtained having activity equivalent to or greater than the wild type enzyme are definitely novel enzymes which are insusceptible to oxidation by treatment with hydrogen peroxide, etc.

The first process is referred to as "a process for producing an enzyme free from sulfur-containing amino acids by combined mutation".

The enzyme free from sulfur-containing amino acids having activity equivalent to or greater than the wild type enzyme according to the present invention can be also prepared in accordance with the following second process.

$A_1/MA_1$  mutant is prepared in accordance with the first process.

Subsequently, double mutants (a maximum of 18 types) are respectively prepared in the same manner as described above wherein the sulfur-containing amino acid at  $A_2$  of  $A_1/MA_1$  mutant is substituted with an amino acid other than sulfur-containing amino acid (a maximum of 18 types). Enzyme activities are then inspected.

When inspecting the activities of the double mutants, mutants having activities equivalent to or greater than that of the wild type are found. From among the double mutants, a maximum of 3 double mutants having the highest activity are selected.

Triple mutants are then prepared respectively (a maximum of  $3 \times 18 = 54$  types) by substituting sulfur-containing amino acids at each A3 of the obtained double mutants by amino acids other than sulfur-containing amino acids (a maximum of 18 types). Enzyme activities thereof are inspected.

When inspecting the activities of the triple mutants, mutants with activity equivalent to or greater than that of the wild type are found.

A quadruple mutant, ..., multiple number of  $n$  mutant are prepared in the same manner as described above. The last multiple number of  $n$  mutant is the contemplated enzyme, which does not contain a sulfur-containing amino acid.

For convenience of description, the order of the sites to be varied is respresented as A1, A2, ..., An, however, the order of mutation is properly selected from any one of ( $n!$  types) permutations and combinations.

The second process is referred to as "a process for producing an enzyme free from sulfur-containing amino acid by stepwise mutation".

In the "process for producing an enzyme free from sulfur-containing amino acids by stepwise mutation", a maximum of  $[4(A1) + 18(A2) + 54 \times (n-2)(A3 \text{ to } An)]$  mutants are inspected.

The enzyme not containing a sulfur-containing amino acid and having activity equivalent to or greater than a wild type enzyme according to the present invention may be obviously prepared by "the process for producing an enzyme free from sulfur-containing amino acids by combined mutation" in partial combination with "the process for producing an enzyme free from sulfur-containing amino acids by stepwise mutation" (referred to as "a process for producing an enzyme free from sulfur-containing amino acids by combined mutation in combination with stepwise mutation").

Information on the amino acid sequence and the base sequence of the target wild type enzyme is sufficient for preparing sulfur atom-free enzyme proteins according to the present invention. For example, in accordance with information on the base sequence, a PCR primer is synthesized. DNA encoding the wild type enzyme can be synthesized by PCR using DNA of cells producing the wild type enzyme, cDNA, or recombinant plasmid DNA as a template. DNA encoding the wild type enzyme can be also prepared by chemical synthesis on the basis of the base sequence. The present invention is performed by subjecting DNA encoding the wild type enzyme to mutation in accordance with the process of the present invention. Accordingly, the present invention is not limited by the gene of the wild type enzyme.

The examples of the present invention provides a typical example of an

oxidoreductase in which a cystein free mutant (abbreviated as AS-DHFR) of *Escherichia coli*-derived dihydrofolate reductase (abbreviated as DHFR) is used as a starting material, 5 methionines contained in AS-DHFR are substituted with other amino acids to prepare DHFR free from sulfur-containing amino acids, having high activity far exceeding the enzyme activity of the wild type DHFR which contains 2 cystein residues and 5 methionine residues. As a typical example of a hydrolytic enzyme, preparation of xylanase free from sulfur-containing amino acids, which has activity equivalent to the wild type enzyme is illustrated, in which 2 methionines contained in *Bacillus subtilis*-derived xylanase are substituted with other amino acids.

SEQ ID NO 1 in the Sequence Listing shows an amino acid sequence of AS-DHFR, SEQ ID NO 2 in the Sequence Listing shows a gene sequence which can be excised with restriction enzyme BamHI and is highly expressible in *Escherichia coli* by introducing into the BamHI site of an appropriate vector for *Escherichia coli*, SEQ ID NO 3 in the Sequence Listing shows the amino acid sequence of *Bacillus subtilis*-derived xylanase, and SEQ ID NO 4 of the Sequence Listing shows a DNA sequence encoding *Bacillus subtilis*-derived xylanase.

DNA of SEQ ID NO 2 in the Sequence Listing can be prepared by using a recombinant plasmid, pTZDHFR20, having a sequence reported by the present inventors (described in *Journal of Biochemistry* vol.117, p.480-488 (1995)) as a template by employing PCR using two primer DNAs, for example, 5'-ggatccttgacaattgtaactat-3' and 5'-ggatccttaacgacgctcaggatt-3'. The DNA can also be prepared by a chemical synthesis based on the sequence of SEQ ID NO 2.

DNA of SEQ ID NO 4 in the Sequence Listing can be prepared from chromosome DNA of *Bacillus subtilis* by employing PCR using, for example, a primer DNA indicated by 5'-gtagcacag actactgcaaaat-3' and 5'-taccatacggtaacattcagc-3'. The present invention employs DNA isolated using chromosome DNA (product number D4041, commercially available from Sigma) as a chromosome DNA of *Bacillus subtilis*. The DNA can also be prepared by a chemical synthesis based on the sequence of SEQ ID NO 4.

AS-DHFR comprises 159 amino acids. Among those, the 1st, 16th, 20th, 42nd, and 92nd amino acids are methionines. As a process for producing enzymes in which each methionine is substituted with another amino acid, the Examples describe a process employing "a process for producing an enzyme free from sulfur-containing amino acids by a combination of combined mutation and stepwise mutation".

Tables 1 to 5 show the enzyme activities of mutants in which the 1st, 16th, 20th, 42nd, and 92nd methionines of AS-DHFR are substituted with other amino acids, Table

6 shows the enzyme activities of mutants in which the 42nd and 92nd methionines of AS-DHFR are substituted with other amino acids, and Table 7 shows the enzyme activities of mutants in which the 16th and 20th methionines of AS-DHFR are substituted with other amino acids.

Regarding the 1st methionine, three types of mutant were prepared in accordance with the above process. As a result, methionine-alanine was selected as the optimal one (see Table 1). This mutant was named AS-DHFR-A1.

Regarding the 16th, 20th, 42nd, and 92nd methionines of AS-DHFR, a random bases- substituted mutant was prepared using a primer with a sequence in which each ATG, as a codon for methionine, had been replaced with NNY (N represents a base of A, T, G, and C and Y represents a base of T and C). The base sequence and the enzyme activity of the obtained mutant were measured. As a result, alanine, phenylalanine, and asparagine were determined as preferable 16th amino acids; isoleucine, leucine, and valine were determined as preferable 20th amino acids; valine and tyrosine were determined as preferable 42nd amino acids; and phenylalanine and isoleucine were determined as preferable 92nd amino acids (see Tables 2 to 5).

Next, valine and tyrosine as the preferable 42nd amino acids are combined with phenylalanine and isoleucine as the preferable 92nd amino acids to prepare the 42nd and 92nd amino acid substitution mutants of AS-DHFR-A1. As a result of measurement of the base sequence and the enzyme activity of the obtained mutants, a combination of tyrosine as the 42nd amino acid and phenylalanine as the 92nd amino acid exhibited the greatest activity (about three times greater than the wild type enzyme). This mutant was named AS-DHFR-A1-M42Y-M92F (see Table 6).

Subsequently, alanine, phenylalanine, and asparagine as preferable 16th amino acids were combined with isoleucine, leucine, and valine as preferable 20th amino acids to prepare 16th and 20th amino acid substitution mutants of AS-DHFR-A1-M42Y-M92F. The base sequence and the enzyme activity of the obtained mutants were measured. As a result, as shown in Table 7, mutant enzymes, the activity of which exceeded that of the wild type enzyme, was obtained. Among them, the combination of asparagine as the 16th amino acid and leucine as the 20th amino acid exhibited the greatest activity (about twelve times greater than the wild type enzyme). This mutant was named ANLYF (based on the single letter representation of the mutations at each methionine site M1MA, M16N, M20L, M42Y, and M92F of AS-DHFR. The same representation is employed hereinafter).

A total of nine mutants including AFLYF, AFIYF, ANIYF, AFVYF, and ANVYF were obtained as sulfur atom-free mutants having activity greater than the wild

type enzyme in addition to ANLYF.

This result indicates that there are many possibilities for sulfur atom-free enzyme proteins. Conversion of the wild type enzyme sequence in accordance with the process of the present invention can prepare a sulfur atom-free enzyme having activity greater than the wild type enzyme. Thus, the usability of the present invention was demonstrated.

Xylanase comprises 185 amino acids with the 158th and the 169th amino acids being methionine. As a process for preparing enzymes in which methionine is substituted with other amino acids, in the Examples, a process is described wherein "a process for producing an enzyme free from sulfur-containing amino acids by stepwise mutation" is employed.

Specifically, a fusion protein was prepared wherein a carboxy terminal of the ANLYF and an amino terminal of xylanase were linked with a sequence, Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly (this was named NL-xylanase). Neither the ANLYF portion in NL-xylanase nor the linker region contained methionine and cysteine. Fusion with ANLYF allows selection of transformants into which NL-xylanase mutant fusion genes have been introduced on the basis of trimethoprim resistance and by measuring xylanase activity and DHFR activity and calculating the value [xylanase activity]/[DHFR activity], it is possible to compare activity of mutant xylanase with wild type xylanase activity without measuring the protein amount of NL-mutant xylanase.

A random bases-substituted mutant was prepared using a primer having a sequence in which "atg" as a codon for the 158th methionine in the xylanase portion of NL-xylanase was changed to "nny" (n represents a base for a, c, g, t and y represents a base for t, c). The base sequence and enzyme activity of the prepared mutant were measured and as a result, a mutant, which had been substituted to leucine, exhibited 127% activity of the wild type. This mutant was named NL-xylanase (M158L).

Subsequently, a random bases-substituted mutant was prepared using a primer having a sequence in which "atg" as a codon for the 168th methionine in xylanase portion of NL-xylanase (M158L) was changed to "nny" (n represents a base for a, c, g, t and y represents a base for t, c). The base sequence and the enzyme activity of the prepared mutant were measured. As a result, a mutant, which had been substituted to isoleucine, exhibited 151% activity of the wild type. This mutant was denoted NL-xylanase (M158L, M169I).

Neither the DHFR portion, the linker region, nor the xylanase of the obtained NL-xylanase (M158L, M169I) contained the sulfur-containing amino acids, cysteine or

methionine. In spite of that, the enzyme activity of DHFR and that of xylanase had greater activities than corresponding wild type enzymes respectively.

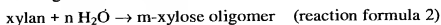
DHFR is a so-called oxidoreductase which catalyzes an oxidation-reduction reaction requiring a nicotinamide coenzyme. Xylanase is a so-called hydrolytic enzyme which catalyzes a hydrolysis reaction of high molecular polysaccharides. Two examples of the present invention demonstrate that, in enzymes catalyzing completely different reactions, i.e., an oxidation-reduction reaction and a hydrolysis reaction, enzymes can be prepared with activity exceeding that of wild type enzymes produced by organic evolution, containing no sulfur-containing amino acids at all. This indicates the existence of proteins having activity exceeding or equivalent to the activity of a wild type enzyme in proteins wherein all the sulfur-containing amino acids contained in the wild type enzyme are substituted with other amino acids. The present invention provides an assured process for being led to such enzymes free from sulfur-containing amino acid. Accordingly, the present invention obviously includes all the enzymes free from sulfur-containing amino acid which can be prepared by the production process of enzymes free from sulfur-containing amino acid described in the present specification.

The dihydrofolate reductase according to the present invention catalyzes the reaction represented by the following reaction formula 1:

$$\text{dihydrofolic acid} + \text{NADPH} \rightarrow \text{tetrahydrofolic acid} + \text{NADP}^+ \quad (\text{reaction formula 1}).$$

Activity of dihydrofolate reductase can be traced by a decrease in absorbance at 340 nm caused by a decrease in substrate caused by reaction. According to the present invention, the enzyme reaction solution comprises 50 mM phosphate buffer (pH 7), 0.1 mM NADPH, 0.05 mM dihydrofolic acid, 12 mM 2-mercaptoethanol, and a suitable amount of enzyme. One ml of enzyme reaction solution was collected in a cuvette for a spectrophotometer. A reaction was initiated with the addition of the enzyme solution and change in amount of absorbance at 340 nm per minute was measured. The quantity was taken as an indicator of activity.

Xylanase according to the present invention catalyzes the reaction represented by the following reaction formula 2:



The activity of xylanase can be observed by measuring an increase of reducing power derived from a reducing terminus of the prepared oligoxylose in accordance with the Nelson-Somogyi method. According to the present invention, the enzyme reaction solution comprises 50 mM sodium acetate buffer (pH 6.0), 2 mg/ml oat derived-xylane, and a proper amount of enzyme. The enzyme reaction solution (0.25 ml) was poured

into a test tube. A reaction was initiated with the addition of the enzyme solution and the reaction was performed for 10 min. After 0.25 ml of copper-alkali reagent was added, the reaction solution was retained in boiled water for 10 min and then cooled to room temperature. Thereafter, 0.25 ml of arsenic- molybdic acid reagent was added, thereby coloring the solution. The intensity of coloring was measured by absorbance at 500 nm to determine a difference between the measured value and the absorbance of the control sample. The obtained value was adopted as an index for enzyme activity.

It is well known that various processes are developed and used for measuring enzyme activity. Thus, the present invention is obviously not limited by a process for measuring the subject enzyme activity.

The sulfur atom-free dihydrofolate reductase and a fused protein of dihydrofolate reductase-xylanase prepared according to the present invention do not contain in the sequences thereof, cysteine and methionine which are easily oxidized by hydrogen peroxide. Therefore, treatment with 0.1 M highly concentrated hydrogen peroxide water does not result in oxidation, and resistance against oxidation is significantly enhanced. For either dihydrofolate reductase or a fused protein of dihydrofolate reductase-xylanase, in the case of a sulfur atom-containing enzyme, treatment with 0.1 M hydrogen peroxide water oxidizes all the sulfur molecules, thereby lowering the enzyme activity to about one tenth or below. It is verified that enhancement of an antioxidative property in this manner contributes to the stability of enzyme traits.

#### BEST MODE TO CARRY OUT THE INVENTION

The present invention will be described with reference to the following examples, though the present invention is not limited to these specific examples only.

In the examples, DNA amplification by PCR, DNA cleavage by restriction enzymes, T4-DNA ligase, DNA linkage by transformation to *Escherichia coli* were carried out in accordance with a standard protocol attached to a commercially available PCR kit, restriction enzymes, T4-DNA ligase, and *Escherichia coli* competent cells. [Example 1] Preparation of sulfur atom-free dihydrofolate reductase

In the present example, AS-DHFR was used as a sulfur atom-containing dihydrofolate reductase. Properties of AS-DHFR such as stability of enzyme activity are substantially in conformity with the wild type enzyme (described in M. Iwakura, B.E. Jones, J. Luo, & C.R. Matthews, *J. Biochemistry*, 117, 480-488 (1995)).

The amino acid sequence of AS-DHFR and the base sequence of genes thereof are shown in SEQ. ID NO 1 in the Sequence Listing and SEQ. ID NO 2 in the Sequence

Listing, respectively.

Genes of AS-DHFR are incorporated in a plasmid which is named "pTZDHFR20" (described in M. Iwakura, B.E. Jones, J. Luo, & C.R. Matthews, J. Biochemistry, 117, 480-488 (1995)).

Two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagtaactattgtataatgtattc-3' and 5'-ggggatcccttatgcacagccaccgccaccacgacgctcgaggatttcg-3', were used, and amplification performed by PCR using pTZDHFR20 as a template, thereby preparing a gene sequence which is able to be cleaved by a restriction enzyme BamHI and to express AS-DHFR (DNA1).

The DNA sequence amplified by PCR was used as a template to prepare genes wherein methionine as the 1st amino acid was varied to L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline. In order to prepare the genes, DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagtaactattgtataatgtattc-3' and 5'-cgcaatcacagtcatgncatggaagttcctctctttccggatt-3' (n represents a base of a, c, g or t) (DNA2) and DNA, amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggaggaaacttccatgncatcagctgattcgccgctagcggtagat-3' (n represents a base of a, c, g or t) and 5'-ggggatcccttatgcacagccaccgccaccacgacgctcgaggatttcg-3' (DNA3) were prepared, and then, DNA2 was mixed with DNA3 in equal amounts, followed by preparation of DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagtaactattgtataatgtattc-3' and 5'-ggggatcccttatgcacagccaccgccaccacgacgctcgaggatttcg-3' (DNA4).

DNA4 was cleaved by BamHI, and then combined with a commercially available plasmid vector pUC19 which had been cleaved by BamHI, and they were ligated using T4-DNA ligase to prepare a recombinant plasmid. The obtained recombinant plasmid was used to transform Escherichia coli JM109 strain and a mutant growing in agar medium 1 (containing 5 g of salt, 5 g of yeast extract, 8 g of trypton, 100 mg of sodium ampicillin, 50 mg of trimethoprim, and 15 g of agar, per liter) was selected. In respect of 15 colonies formed in agar medium 1, plasmids were separated, and the base sequences thereof were determined, thereby isolating a transformant comprising a gene in which methionine as the 1st amino acid had been varied to L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline. Each of the isolated transformants was cultured at 37 degree overnight in medium 1 (containing 5 g of salt, 5 g of yeast extract, 8 g of trypton, and 100 mg of sodium ampicillin, per liter). Absorbance at 660 nm was adjusted to 1, bacterial cells were collected from 1



ml of the culture solution, suspended in 0.2 ml 10 mM phosphate buffer (pH 7.0), subjected to sonication, and then centrifuged. The obtained supernatant was inspected for its DHFR enzyme activity. The results are shown in Table 1.

In the Table below, DHFR activity is indicated by % with the activity of the wild type enzyme being 100%.

Table 1	
(Types of mutation)	(DHFR activity)
L-methionine-L-alanine (AS-DHFR-A1)	103
L-methionine-L-serine	98
L-methionine-L-proline	89

The results indicate that L-methionine-L-alanine is suitable as a mutant for the 1st amino acid (this mutant is referred to as AS-DHFR-A1, and a recombinant plasmid including AS-DHFR-A1 gene is referred to as pAS-DHFR-A1). AS-DHFR-A1 was highly purified, and the sequence of the amino terminus was inspected. As a result, 99% or more of the amino acids were alanine, and methionine resulting from the initiation codon on the gene sequence had been substantially completely excised by methionyl-aminopeptidase during expression in *Escherichia coli*.

Regarding the 16th, 20th, 42nd, and 92nd methionines of AS-DHFR, a random bases-substituted mutant was prepared using a primer having a sequence in which atg, a codon of methionine, has been varied to nny (n represents a base of a, c, g or t, r represents a base of a or g, and y represents a base of c or t). The base sequence and the enzyme activity of the obtained mutant were measured.

In preparation of a mutant for the 16th methionine, by using DNA1 as a template, DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttactattgttataatgtattc-3' and 5'-ggcatggcggttttcnngccgataacgcgatctaccgcta-3' (DNA5) and DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-gatcgcgttatcgccnnygaaaacgccatgccatggaac-3' and 5'-ggggatcccttatgacacgccaccgcaccagacgctcgaggatttcg-3' (DNA6) were prepared. Subsequently, DNA5 was mixed with the same amount of DNA6 and then, DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttactattgttataatgtattc-3' and 5'-ggggatcccttatgacacgccaccgcaccagacgctcgaggatttcg-3' (DNA7) was prepared.

After cleaving DNA7 with BamHI, the same procedures as described above were performed and colonies growing in agar medium 1 were inspected. The results are shown in Table 2.

Table 2

[Types of mutation] (16th amino acid)	[DHFR activity]
L-alanine	99
L-aspartic acid	9.5
L-phenylalanine	234
glycine	31
L-histidine	44
L-isoleucine	52
L-leucine	30
L-asparagine	100
L-proline	1.7
L-arginine	60
L-serine	95
L-threonine	61
L-valine	34
L-tyrosine	73

This result indicates that alanine, phenylalanine, and asparagine are suitable as the 16th amino acid.

In preparation of a mutant for the 20th methionine, using DNA1 as a template, DNA amplified by PCR using two chemically-synthesized primer DNAs:

5'-ggggatcctcttgacaattagtaactattgttataatgtattc-3' and

5'-aggcaggttccatggrnngcgcttttccatgccgataac-3' (DNA8) and DNA amplified by PCR using two chemically-synthesized primer DNAs:

5'-ggcatggaaaacgccnnyccatggaacctgcctgccgatc-3' and

5'-ggggatcccttatgcacagccaccgccaccacgacgctcgaggatttcg-3' (DNA9) were prepared.

Subsequently, DNA8 was mixed with the same amount of DNA9 and then, DNA amplified by PCR using two chemically-synthesized primer DNAs:

5'-ggggatcctcttgacaattagtaactattgttataatgtattc-3' and

5'-ggggatcccttatgcacagccaccgccaccacgacgctcgaggatttcg-3' (DNA10) was prepared.

After cleaving DNA 10 with BamHI, the same procedures as described above were

performed and colonies growing in agar medium 1 were inspected. The results are shown in Table 3.

Table 3	
[Types of mutation] (20th amino acid)	[DHFR activity]
L-aspartic acid	3.7
glycine	0.8
L-histidine	1.7
L-isoleucine	182
L-leucine	283
L-proline	1.2
L-arginine	2
L-serine	20
L-threonine	63
L-valine	122
L-tyrosine	46

This result indicates that isoleucine, leucine, and valine are suitable as the 20th amino acid.

In preparation of a mutant for the 42nd methionine, by using DNA1 as a template, DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttaactatttggtataatgtattc-3' and 5'-ccagggtatggcgcccrnnaatcacgggtttatttaagggtg-3' (DNA11) and DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-aataaaccctgtgattnnnyggcgccatacctgggaatcaa-3' and 5'-ggggatccctatgcacagccaccgcaccagcgtcgaggatttcg-3' (DNA12) were prepared. Subsequently, DNA11 was mixed with the same amount of DNA12 and then, DNA obtained through amplification by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttaactatttggtataatgtattc-3' and 5'-ggggatccctatgcacagccaccgcaccagcgtcgaggatttcg-3' (DNA13) was prepared. After cleaving DNA13 with BamHI, the same procedures as described above were performed and colonies growing in agar medium 1 were inspected. The results are shown in Table 4.

Table 4

[Types of mutation] (42nd amino acid)	[DHFR activity]
L-alanine	63
L-phenylalanine	49
glycine	5.5
L-threonine	14
L-valine	71
L-tyrosine	167

This result indicates that valine and tyrosine are suitable as the 42nd amino acid.

In preparation of a mutant for the 92nd methionine, by using DNA1 as a template, DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagtaactattgtataatgtattc-3' and 5'-tccgccccaatcacrnngattttcgtacgtcacctgcg-3' (DNA14) and DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-gacgtaccagaaatcnnygtgattggcggggacgcgttt-3' and 5'-ggggatcctcttgacacgccaccgccaccacgacgctcgaggatttcg-3' (DNA15) were prepared. Subsequently, DNA14 was mixed with the same amount of DNA15 and then, DNA obtained through amplification by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagtaactattgtataatgtattc-3' and 5'-ggggatcctcttgacacgccaccgccaccacgacgctcgaggatttcg-3' (DNA16) was prepared. After cleaving DNA16 with BamHI, the same procedures as described above were performed and colonies growing in agar medium 1 were inspected. The results are shown in Table 5.

Table 5

[Types of mutation] (92nd amino acid)	[DHFR activity]
L-aspartic acid	34
L-phenylalanine	69
glycine	0.2
L-isoleucine	37
L-arginine	0.5

This result indicates that phenylalanine and isoleucine are suitable as the 92nd

amino acid.

Next, valine or tyrosine as the preferable 42nd amino acid of AS-DHFR-A1 was combined with phenylalanine or isoleucine as the preferable 92nd amino acid to prepare the 42nd and 92nd amino acid-substituted mutants of AS-DHFR-A1. The base sequence and the enzyme activity of the obtained mutants were measured.

Using pAS-DHFR-A1 as a template, DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagtaactattgttataatgtattc-3' and 5'-ccaggatgagcgcccrwmaatcacgggtttatttaagggtg-3' (r represents a base of a or g, w represents a base of a or t, and m represents a base of a or c, the same shall apply hereinafter) (DNA17) and DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-aataaaccttgatgkwyggcgccatactgggaatcaa-3' (k represents a base of g or t, w represents a base of a or t, and y represents a base of c or t, the same shall apply hereinafter) and 5'-ggggatcccttatgcacagccaccgccaccagcagctcgaggatttcg-3' (DNA18) were prepared. Subsequently, DNA17 was mixed with the same amount of DNA18, and then, DNA was prepared by amplifying by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagtaactattgttataatgtattc-3' and 5'-ggggatcccttatgcacagccaccgccaccagcagctcgaggatttcg-3' (DNA19).

The obtained DNA19 was used as a template. DNA, amplified by PCR using two chemically-synthesized primer DNAs:

5'-ggggatcctcttgacaattagtaactattgttataatgtattc-3' and

5'-tcgcccgcgaatcawgatgttcgtgtagctcactgcg-3', was prepared (DNA20) and DNA, amplified by PCR using two chemically-synthesized primer DNAs:

5'-gacgtaccagaaatcwtygtgattggcgcgagcgcgttt-3' and

5'-ggggatcccttatgcacagccaccgccaccagcagctcgaggatttcg-3' (DNA21), was prepared.

Subsequently, DNA20 was mixed with the same amount of DNA21 and then DNA was prepared in which two chemically-synthesized primer DNAs:

5'-ggggatcctcttgacaattagtaactattgttataatgtattc-3 and

5'-ggggatcccttatgcacagccaccgccaccagcagctcgaggatttcg-3' were used and amplified by PCR (DNA22).

After cleaving DNA22 with BamHI, the same procedures as described above were performed and colonies growing in agar medium 1 were inspected. The results are shown in Table 6.

Table 6

[Types of mutation]

(42nd amino acid)

[DHFR activity]

(92nd amino acid)

L-tyrosine	L-phenylalanine	320
L-tyrosine	L-isoleucine	61.3
L-valine	L-phenylalanine	102
L-valine	L-isoleucine	58.9

The result shows that a combination of tyrosine as the 42nd amino acid and phenylalanine as the 92nd amino acid exhibited the greatest activity (about three times greater than the wild type enzyme). This mutant is referred to as AS-DHFR-A1-M42Y-M92F. The recombinant plasmid including genes of AS-DHFR-A1-M42Y-M92F is named pAS-DHFR-A1-M42Y-M92F.

Alanine, phenylalanine, and asparagine as the preferable 16th amino acid of AS-DHFR-A1-M42Y-M92F were combined with isoleucine, leucine, and valine as the preferable 20th amino acid to prepare the 16th and 20th amino acid substitution mutants of AS-DHFR-A1-M42Y-M92F. The base sequence and the enzyme activity of the obtained mutants were measured.

DNA, amplified by PCR using three chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttaactattgttataatgtattc-3', 5'--aggcaggttccacggrkwggcgcttttctgcccataacgcgatctaccg-3', and 5'-aggcaggttccacggrkwggcgcttttctgcccataacgcgatctaccg-3' (DNA23) and DNA, amplified by PCR using three chemically-synthesized primer DNAs: 5'-gatcgcgttatcggcarygaaaacgccwmyccgtggaacctgcctgccga-3', 5'-gatcgcgttatcggcgcagaaaacgccwmyccgtggaacctgcctgccga-3', and 5'-ggggatcccttatgcacagccaccgccaccacgacgctcgaggatttcg-3' (DNA24) were prepared using pAS-DHFR-A1-M42Y-M92F as a template. Subsequently, DNA23 was mixed with the same amount of DNA24. Thereafter, DNA, obtained through amplification by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttaactattgttataatgtattc-3' and 5'-ggggatcccttatgcacagccaccgccaccacgacgctcgaggatttcg-3' was prepared (DNA25).

After cleaving DNA25 with BamHI, the same procedures as described above were performed and colonies growing in agar medium 1 were inspected. The results are shown in Table 7.

Table 7

[Types of mutation]		[DHFR activity]
(16th amino acid)	(20th amino acid)	
L-alanine	L-isoleucine	240

L-alanine	L-leucine	579
L-alanine	L-valine	102
L-phenylalanine	L-isoleucine	176
L-phenylalanine	L-leucine	745
L-phenylalanine	L-valine	163
L-asparagine	L-isoleucine	102
L-asparagine	L-leucine	989
L-asparagine	L-valine	127

The results indicate that the combination of asparagine as the 16th and leucine as the 20th has the greatest activity (about 10 times greater than that of the wild type enzyme). This mutant is referred to as ANLYF. The recombinant plasmid including ANLYF genes was named pANLYF. Other 8 mutants had activities exceeding the wild type enzyme. This result indicates that there are many possibilities for sulfur atom-free enzyme proteins.

SEQ ID NO 5 in the Sequence Listing shows the amino acid sequence of ANLYF and SEQ ID NO 6 in the Sequence Listing shows the ANLYF gene sequence which is excisable by restriction enzyme BamHI and highly expressible in *Escherichia coli* by introduction thereof into a BamHI site with an appropriate vector.

*Escherichia coli* containing pANLYF was cultured in 3L of medium (containing 15 g of salt, 15 g of yeast extract, 24 g of trypton, and 30 mg of sodium ampicillin) at 37°C overnight to obtain about 10 g of cells on a wet basis. A cell-free extract from the cells was subjected to purification by streptomycin sulfate treatment, ammonium sulfate fractionation, methotrexate affinity chromatography, and DEAE Toyopearl chromatography to purify proteins to homogeneity. Thus, about 100 mg of homogeneous ANLYF was obtained. As a result of N-terminal analysis, the amino-terminal sequence of ANLYF was

L-alanine-L-isoleucine-L-serine-L-leucine-L-isoleucine and was a sequence with an initiation codon which is corresponding to L-methionine being removed therefrom. 1 mg of ANLYF obtained through purification was allowed to stand overnight at room temperature in 10 mM phosphate buffer (pH 7.0) containing 0.1M hydrogen peroxide water. Molecular weight was measured, and as a result, the measured value was exactly same as the molecular weight of untreated ANLYF, i.e., 17,905 (calculated value: 17,903). Enzyme activity was also completely unchanged. In contrast, AS-DHFR was subjected to the same treatment, and as a result, molecular weight before treatment with hydrogen peroxide water was 17,954 (calculated value: 17,950) and the

molecular weight after treatment with hydrogen peroxide water was 18,034. This indicates that all methionine residues were oxidized to methionine sulfoxide. The enzyme activity of AS-DHFR was lowered to about one fifth as a result of oxidation of all methionine residues. Thus, an antioxidative property was imparted by the exclusion of sulfur atoms.

[Example 2] Preparation of sulfur atom-free dihydrofolate reductase-xylanase fusion enzyme

For the preparation of a fused gene of dihydrofolate reductase and xylanase, as a gene portion of dihydrofolate reductase, a sequence corresponding to 1 to positions No. 1 to No. 560 in SEQ ID NO 6 of the Sequence Listing was used. As a xylanase gene portion, DNA was prepared by amplifying by PCR using two chemically-synthesized primer DNAs: 5'-gctagcacag actactggcaaat-3' and 5'-ttaccatcacgtaacattcgacg-3' (DNA26) and using chromosome DNA as a template, which is commercially available from Sigma as chromosome DNA of *Bacillus subtilis* (product number D4041). The prepared DNA was linked with a base sequence corresponding to the codon for Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly to prepare a fusion gene.

At the outset, DNA was prepared by PCR amplification using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttaactattgttataatgtattc-3' and 5'-gccgccaccaccgcgaccgccaccacgacgctcgaggatttcgaacgaata-3', and using pNLYF as a template (DNA27). Subsequently, DNA26 was used as a template to prepare DNA by PCR amplification using two chemically-synthesized primer DNAs: 5'-gggtggcggtggctcgggtgggtggcgcgctagcacagactactggcaaaattggactgat-3' and 5'-ggggatccttaccatcacggtaacattcgacgagccactactttga-3' (DNA28). DNA27 was mixed with the same amount of DNA28, thereafter, two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttaactattgttataatgtattc-3' and 5'-ggggatccttaccatcacggtaacattcgacgagccactactttga-3' were used to prepare DNA obtained through amplification by PCR (DNA29).

DNA29 was cleaved by BamHI, and then, 10 colonies growing in agar medium 1 were collected in the same manner as described above, a recombinant plasmid was isolated, the base sequence of the portion included in the plasmid was inspected, and a recombinant plasmid which included the subject sequence was isolated, and named pNLXYL-wt.

SEQ ID NO: 7 of the Sequence Listing shows the amino acid sequence of dihydrofolate reductase-xylanase fusion enzyme. SEQ ID NO: 8 of the Sequence



Listing shows the gene sequence of dihydrofolate reductase-xylanase fusion enzyme which can be cleaved by restriction enzyme BamHI and is highly expressible in *Escherichia coli* by introduction thereof into a BamHI site with an appropriate vector. A sequence corresponding to positions No. 1 to No. 159 in SEQ ID NO 7 in the Sequence Listing is the dihydrofolate reductase (ANLYF mutant) portion, the sequence corresponding to positions No. 160 to No. 168 is a linker sequence for linking dihydrofolate reductase to xylanase without difficulties, and the sequence corresponding to positions No. 169 to No. 353 is a sequence of the wild type xylanase.

Xylanase comprises 185 amino acids. Among them, the 158th and the 169th amino acids are methionine. Thus, in dihydrofolate reductase-xylanase fused enzyme, the 326th and the 337th sequences are methionines. In this dihydrofolate reductase-xylanase fusion enzyme, no other sulfur-containing amino acids exist besides the above.

In preparation of a 326th methionine mutant of a dihydrofolate reductase-xylanase fusion enzyme, pNLXYL-wt was used as a template to prepare DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctctgacaattagttaactattgttataatgtattc-3' and 5'-gacttgtaagcccaattactgccagattgnntccatgcgtcttccatcggtt-3' (DNA30). DNA30 was used as a template to prepare DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctctgacaattagttaactattgttataatgtattc-3' and 5'-actttgatctctctgtcgccatgacttggtgaagcccaattactgccagatt-3' (DNA31). Further, DNA31 was used as a template to prepare DNA amplified by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctctgacaattagttaactattgttataatgtattc-3' and 5'-ggggggatccttaccatcaggtgaacattgcagagccactacttgatactctctgtctgc-3' (DNA32). After cleaving DNA32 with BamHI, the same procedures as described above were performed and colonies growing in agar medium 1 were inspected. The xylanase activity of each mutant was also inspected.

From the measured xylanase activity and dihydrofolate reductase activity, the value  $[\text{xylanase activity}]/[\text{DHFR activity}]$  was calculated, and compared to value  $[\text{xylanase activity}]/[\text{DHFR activity}]$  exhibited by cells, which retain pNLXYL-wt (this is determined as the activity of the wild type enzyme). The results are shown in Table 8. In the following table,  $[\text{xylanase activity}]/[\text{DHFR activity}]$  is indicated by % with the activity of the wild type enzyme being 100%.

Table 8

[Type of mutation]	$[\text{xylanase activity}]/[\text{DHFR activity}]$
--------------------	---

(326th amino acid)

L-aspartic acid	0.0
L-phenylalanine	112
L-histidine	5.9
L-isoleucine	66
L-leucine	127
L-asparagine	100
L-serine	7.9
L-threonine	0.7
L-valine	58

This result indicates that leucine is suitable as the 326th amino acid.

This mutant was named NL-xylanase (M158L). A recombinant plasmid which includes NL-xylanase (M158L) gene was named pNLXYL-M158L.

In preparation of a 337th methionine mutant of NL-xylanase (M158L), pNLXYL-M158L was used as a template, and DNA was prepared by amplifying by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttatattgttataatgtattc-3' and 5'-actttgatactctctgtcgcgnggacttggttaagcccaattactgccagatt-3' (DNA33). DNA33 was used as a template and DNA was prepared by amplifying by PCR using two chemically-synthesized primer DNAs: 5'-ggggatcctcttgacaattagttactattgttataatgtattc-3' and 5'-ggggggatccttaccatacggtaacattcgacgagccactactttgatactctctgtcgc-3' (DNA34). After cleaving DNA34 with BamHI, the same procedures as described above were performed and colonies growing in agar medium 1 were inspected. The xylanase activity of each mutant was also inspected. The results are shown in Table 9.

Table 9

[Type of mutation] (337th amino acid)	[Xylanase activity]/[DHFR activity]
L-aspartic acid	0.1
Glycine	0.0
L-histidine	111
L-isoleucine	151
L-asparagine	26
L-proline	1.7
L-arginine	0.0

L-threonine	0.7
L-tyrosine	2.1

This result indicates that isoleucine is suitable as the 326th amino acid.

This mutant was named NL-xylanase (M158L, M169I). A recombinant plasmid which includes NL-xylanase (M158L, M169I) genes was named pNLXYL-LI.

SEQ ID NO 9 in the Sequence Listing shows NL-xylanase (M158L, M169I), and SEQ ID NO 10 in the Sequence Listing shows NL-xylanase (M158L, M169I) which can be cleaved by restriction enzyme BamHI and is highly expressible in *Escherichia coli* by introduction into a BamHI site with an appropriate vector.

*Escherichia coli* containing pNLXYL-LI was cultured in 3 L of medium (containing 15 g of salt, 15 g of yeast extract, 24 g of trypton, and 30 mg of sodium ampicillin) at 37°C overnight to obtain about 10 g of cells on a wet basis. Cell-free extract from the cells was subjected to purification by a streptomycin sulfuric acid treatment, ammonium sulfate fraction, methotrexate affinity chromatography, and DEAE Toyopearl chromatography to purify proteins to homogeneity. Thus, about 30 mg of homogeneous NL-xylanase (M158L, M169I) was obtained. As a result of N-terminal analysis, amino-terminal sequence of NL-xylanase (M158L, M169I) was L-alanine-L-isoleucine-L-serine-L-leucine-L-isoleucine-- and was a sequence from which an initiation codon which is corresponding to L-methionine had been removed. One mg of NL-xylanase (M158L, M169I) obtained through purification was allowed to stand overnight at room temperature in 10 mM phosphate buffer (pH 7.0) containing 0.1M hydrogen peroxide water. Molecular weight was measured, and as a result, the measured value was exactly same as the molecular weigh of untreated NL-xylanase (M158L, M169I), i.e., 38,775 (calculated value: 38,773). The enzyme activity was also completely unchanged. In contrast, dihydrofolate reductase-xylanase fused enzyme before mutation was subjected to the same treatment, and as a result, the molecular weight before treatment with hydrogen peroxide water was 38,813 (calculated value: 38,809) and the molecular weight after treatment with hydrogen peroxide water was 38,845. This indicates that all methionine residues were oxidized to methionine sulfoxide. The enzyme activity of xylanase was lowered to about one third as a result of oxidation of all methionine residues. Thus, an antioxidative property was imparted by the exclusion of sulfur atoms.

#### INDUSTRIAL APPLICABILITY

The present invention provides enzyme proteins, which retain the activity of

the wild type enzyme while having resistance against oxidation caused by hydrogen peroxide and the like, and being chemically stable and a process for producing the same. The enzyme proteins according to the present invention have stable properties, and thus, can be widely used in applications such as biosensors and bioreactors. Therefore, the enzyme proteins according to the present invention are valuable from a practical point of view.

## CLAIMS

1. A sulfur atom-free enzyme protein comprising 18 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; and L-tryptophan.

2. The sulfur atom-free enzyme protein according to claim 1 which retains the activity of the original enzyme protein and has oxidation resistance, wherein L-cysteine and L-methionine residues in enzyme proteins comprising 20 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; L-tryptophan; L-cysteine; and L-methionine, are substituted with 18 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; and L-tryptophan.

3. The sulfur atom-free enzyme protein according to claim 2 wherein amino acid substitution is carried out by site-directed mutagenesis using synthetic DNA.

4. The sulfur atom-free enzyme protein according to any one of claims 1 to 3 wherein the enzyme activity is oxidation-reduction activity or hydrolysis activity.

5. The sulfur atom-free enzyme protein according to any one of claims 1 to 4, which retains the activity of dihydrofolate reductase and has oxidation resistance.

6. The sulfur atom-free enzyme protein according to any one of claims 1 to 4, which retains the activity of xylanase and has oxidation resistance.

7. A method of producing a sulfur atom-free enzyme protein prepared by combined mutation comprising the following steps:

- (1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein consisting of a total length of m amino acids, and comprising n number of sulfur atom-containing amino acids (sulfur-containing amino acids), wherein a position of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), by L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline codon, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting the protein with the highest activity, thereby obtaining a substitution mutant A1/MA1;

- (2) preparing a mutant gene in which codons encoding sulfur-containing amino acids at another sites  $A_i$  ( $i = 2$  to  $n$ ) are substituted with codons encoding another amino acids among the 18 types of amino acid according to claim 1, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting  $p$  number of mutant enzyme proteins having enzyme activity, thereby obtaining substitution mutants  $A_i/B_{ij}$  ( $j = 1$  to  $p$ );
- (3) selecting a maximum of 3 substitution mutants having the highest activity  $A_i/B_{i1}$ ,  $A_i/B_{i2}$ , and  $A_i/B_{i3}$ , wherein activity decreases in order,  $A_i/B_{i1} > A_i/B_{i2} > A_i/B_{i3} > \dots > A_i/B_{ip}$ ; and
- (4) selecting substitution mutants having activity in respect of sulfur-containing amino acids at all sites  $A_i$  ( $i = 2$  to  $n$ ) in the same manner as (2) and (3) above, preparing a maximum of  $3 \times (n-1)$  mutants being all combinations of these mutants with the mutant of  $A1/MA1$ , measuring the enzyme activity thereof, and obtaining a mutant enzyme protein having activity greater than or equivalent to the original enzyme protein.

8. A method of producing a sulfur atom-free enzyme protein prepared by stepwise mutation comprising the following steps:

- (1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein consisting of a total length of  $m$  amino acids, and comprising  $n$  number of sulfur atom-containing amino acids (sulfur-containing amino acids), wherein a position of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), by L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline codon, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting the protein with the highest activity, thereby obtaining a substitution mutant  $A1/MA1$ ;
- (2) preparing a mutant gene in which codons encoding sulfur-containing amino acids at  $A2$  of  $A1/MA1$  mutant is substituted with a codon encoding another amino acid being one of the 18 types of amino acid according to claim 1, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained double mutant enzyme protein, and selecting a maximum of 3 triple mutants with the highest activity;
- (3) preparing a mutant gene in which a codon encoding a sulfur-containing amino acid at each  $A3$  of the obtained double mutants is substituted with a

codon encoding another amino acid being one of the 18 types of amino acid according to claim 1, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained triple mutant enzyme protein, and selecting a maximum of 3 triple mutants with the highest activity; and

- (4) in the same manner as described above, preparing a quadruple mutant, ..., multiple number of  $n$  mutant, inspecting enzyme activity of the last multiple number of  $n$  mutant, and preparing a mutant enzyme protein having activity greater than or equivalent to that of the original enzyme.

9. The process for producing a sulfur atom-free enzyme protein prepared by the stepwise mutation according to claim 8 wherein the order of stepwise mutation sites is according to any one of ( $n!$  types) permutations and combinations of  $A_1, A_2, \dots, A_n$ .

10. A process for producing a sulfur atom-free enzyme protein wherein, in an enzyme protein consisting of a total length of  $m$  amino acids and comprising  $n$  number of sulfur-containing amino acids, in which a site of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), a process according to claim 7 is adopted at  $k$  number of sites and a process according to claim 9 is adopted at remaining  $n - k$  number of sites.

## ABSTRACT

A sulfur atom-free enzyme protein retaining the activity of the original enzyme protein and having oxidation resistance wherein L-cystein and L-methionine residues of the enzyme protein are substituted with 18 types of L-amino acid residues: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; and L-tryptophan. A enzyme protein retaining the activity of the wild type enzyme while having antioxidation properties against oxidation by hydrogen peroxide, and the like and a process for producing the same.



Attorney's Docket No.: \_\_\_\_\_

**DECLARATION, POWER OF ATTORNEY AND PETITION**

I (We), the undersigned inventor(s), hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I (We) believe that I am (we are) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

SULFUR ATOM-FREE ENZYME PROTEIN

the specification of which

☐ is attached hereto.

☐ was filed on \_\_\_\_\_ as

Application Serial No. \_\_\_\_\_

and amended on \_\_\_\_\_

☒ was filed as PCT international application

Number PCT/JP00/02112

on March 31, 2000

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

I (We) hereby state that I (We) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that I (We) do not know and do not believe that this invention was ever known or used before my invention or discovery thereof, or patented or described in any printed publication in any country before my invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States for more than one year prior to this application; that this invention or discovery has not been patented or made the subject of an inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months before this application.

I (We) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

I (We) hereby claim foreign priority benefits under Section 119(a)-(d) of Title 35 United States Code, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Filing date	Priority claimed
<u>183664/1999</u>	<u>Japan</u>	<u>June 29, 1999</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
<u>                  </u>	<u>                  </u>	<u>                  </u>	<input type="checkbox"/> Yes <input type="checkbox"/> No
<u>                  </u>	<u>                  </u>	<u>                  </u>	<input type="checkbox"/> Yes <input type="checkbox"/> No
<u>                  </u>	<u>                  </u>	<u>                  </u>	<input type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim the benefit under Section 119(e) of Title 35 United States Code,  
of any United States application(s) listed below.

(Application Number)

(Filing Date)

(Application Number)

(Filing Date)

I (We) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, I (We) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
_____	_____	_____
_____	_____	_____
_____	_____	_____

And I (We) hereby appoint: FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P., CUSTOMER NUMBER 22,852

I (We) hereby request that all correspondence regarding this application be sent to the firm of FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P. whose Post office address is: 1300 I Street, N.W., WASHINGTON, D.C. 20005 U.S.A.

I (We) declare further that all statements made herein of my (our) knowledge are true and that all statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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